nature portfolio

Peer Review File

Harnessing intestinal tryptophan catabolism to relieve atherosclerosis in mice

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

This paper addresses the issue of the mechanisms whereby gut inflammation and dysbiosis might specifically contribute to atherosclerosis. The Authors show that the shift of intestinal tryptophan (Trp) metabolism from the production of microbiota-derived indole metabolites and 5-hydroxytryptamine (5-HT, serotonin) towards kynurenine production occurs in mice on high-fat diet (HFD) and is likely protective given that in HFD+HCD fed mice, the specific deletion of IDO in intestinal epithelial cells led to intestinal inflammation, impaired intestinal barrier, and exaggerated atherosclerosis along with augmented T lymphocyte accumulation within atheromatous plaques. This was associated with an increase in 5-HT production and a decrease in indole metabolites activating AhR. Inhibition of intestinal 5-HT production alleviated atherosclerosis and inflammation, while supplementation with an AhR agonist had the opposite effect. The Authors claim they have unraveled a novel function for intestinal IDO in the fine-tuning of Trp metabolism with systemic effects on atherosclerosis.

Comments

Low-density lipoprotein receptor (Ldlr-/-) mice, should be…-deficient mice

Several studies have also demonstrated a correlation between serotonin levels and the severity of atherosclerosis in animal models and human populations. Although the exact mechanisms through which serotonin contributes to atherosclerosis are still being investigated, elevated serotonin levels, either due to increased synthesis or impaired breakdown, have been associated with more extensive plaque formation and increased plaque vulnerability. The key question remains elucidating the environmental factors affecting if and how serotonin exerts pro-inflammatory, pro-thrombotic, and pro-proliferative effects that can contribute to the development and progression of atherosclerosis. In this work the Authors found that IDO1 may regulate serotonin production in that IDO1 deficiency in intestinal epithelial cells was associated with high levels serotonin production contributing to atherosclerosis. However, as N-acetylserotonin, a tryptophan metabolite produced along the serotonin pathway, acts as a positive allosteric modulator of IDO1 and thus increases kynurenine-mediated AhR activation (PNAS 117 (7) 3848-3857) – it needs to be assessed whether IDO1 activity, not modified in the gut upon blockade of TPH1, is instead induced by serotonin outside the intestinal epithelial cells, thus contributing to atherosclerosis. Indeed, in the context of atherosclerosis, the increased activity of IDO1 can contribute to the formation of unstable plaques and promote plaque inflammation. The authors have previously shown that IDO1 regulates immune homeostasis in atherosclerosis and colitis through IL-10 inhibition via the production of kynurenines, such that exogenous supply of kynurenines promoted atherosclerosis in IDO1-deficient mice and its blood levels predict death and recurrent myocardial infarction in patients with coronary artery disease. Here, kynurenine administration failed to increase plaque size in Ldlr-/- IEC IDO KO suggesting that the main pathway responsible for atherosclerosis was not dependent on Kyn or Kyn-derived metabolites. The authors need to explain such a discrepancy and the contingency of kynurenine's molecular activity upon the different experimental settings.
Was IDO1 induced in MOMA2+ macrophages within the atherosclerotic plaques in male Ldlr-/- IEC IDOKO mice?

The absence of IDO in IECs led to a significant increase in 5-HT production and plaque size in Ldlr-/- IEC IDOKO. Blockade of TPH1 was associated with a reduction in plaque size in the aortic sinus and in the thoracic aorta. However, to prove that intestinal 5-HT exerts pro-inflammatory and pro-atherogenic effects, as the authors claim, 5-HT needs to be administered under conditions of TPH1 deficiency. Moreover, a mechanistic explanation as to why 5-HT production increases in conditions of IDO-deficiency would be needed, considering the complex interplay between the IDO/Kyn and the 5-HT pathways at different body sites.

The 16S rDNA sequencing of male Ldlr-/-IEC IDOKO and Ldlr-/-IEC IDO mice fed either HFD+HCD or NCD for 8 weeks revealed significant PCoA and relative abundances differences between the groups of mice associated with indoles production. The authors showed that dysbiosis caused by HDF feeding impacted gut inflammation and atherosclerosis. Who does what, and how? How the production of indoles are unexpectedly decreased under conditions of IDO1 deficiency in IEC? It has been demonstrated that the opposite holds true. The Authors shoud explain the discrepancy. Finally, is not particularly surprising AhR's role in atherosclerosis is not particularly surprising; the novelty would be clarifying how it is affected by either type of diet and/or specifically upon activation vs. inhibition of AhR activity.

Reviewer #2 (Remarks to the Author):

Chajadine et al. investigate the role intestinal tryptophan catabolism in experimental atherosclerosis. They compared high fat (HFD), high cholesterol (HCD) and the combination with normal chow diet (NCD). One big issue arising in the study is that HFD and HCD were purified diets from Ssniff, Germany and the normal chow diet (A03, SAFE, France) was from a vendor in France. This issue makes all comparisons between HFD/HCD vs. NCD highly problematic, because the different diets show substantial differences (e.g. fiber, starch, choline, arginine, tryptophan, etc.) all known confounders for the microbiome, intestinal metabolites, vascular and immune phenotypes. This issue also leads to wrong interpretations on their microbiome and intestinal metabolite data. Nevertheless, several sub studies comparing genetic differences under one diet condition still are valid and interesting. They found that under HFD+HCD, the specific deletion of IDO in intestinal epithelial cells led to intestinal inflammation, impaired intestinal barrier, and exaggerated atherosclerosis along with augmented T lymphocyte accumulation within atheromatous plaques. The phenotype was associated with an increase in 5-HT production and a decrease in indole metabolites. Of note, the Ldlr-/-Rag1-/-IEC IDOKO mouse studies demonstrate a clear role of lymphocytes in the IDO- atheromatous plaque phenotype.

Major critique:

• All NCD vs. other group comparisons should be removed from the paper. Alternatively, the authors could repeat the whole study with an appropriated control diet.
• The authors need to follow the ARRIVE guidelines. One important challenge in science is to improve the reproducibility. Therefore, precise reporting of methodological and technical details is essential, especially since it get more and more obvious that environmental factors are important modifiers of phenotypes. The authors do not provide sufficient information. E.g. the genetic strains including backcrossings is not reported; information on housing conditions (single vs. cage housing including the number of cages) is missing – cage effects have a pivotal role on microbiome; health status for the different lines need to be provided in the supplement, since different pathogen status is known to affect phenotypes, statistical details (e.g. outlier testing, testing for normal distribution, description which tests where used for which subset of experiments need to be described in each legend, etc.) are missing.
• Along this line, the authors have performed experiments after antibiotics treatment. Except "We also subjected some mice to antibiotic (ATB) treatment as described before Ref. 45." and "male mice fed HFD+HCD using a broad-spectrum antibiotic cocktail (ATB) supplemented in the drinking water" no information on the type of antibiotics is given. It is not the task of the reviewer to hunt for this information.
• The microbiome data is superficial and not of high quality. Therefore, it should be deleted. Since the co-housing experiments are performed on the same diet (HFD+HCD), this set of experiment is fine.
• Why do the authors sole report on C2 and C4 and not on C3?
• What are the fecal IEC IDO KO SCFA levels?
• Why do male Ldlr-/-Rag1-/-IEC IDO show already higher plaques size in a similar range as male IEC IDOKO in Fig. 1H?
• The authors found that AhR agonists (IAA, IAld, and tryptamine) were markedly decreased in Ldlr-/- IEC IDO KO and Ldlr-/- IEC IDO mice treated with ATB. Why did they perform the rescue experiment with FICZ and not a natural Ahr ligand such as e.g. IAA?
• What is the role of FICZ on Th17/Treg ratio as well as IL-22?

Reviewer #3 (Remarks to the Author):

The current manuscript reported the key role of intestinal tryptophan catabolism in the development and protection of atherosclerosis of disease. The shift of trytophan catabolism under HFD feeding significantly influenced the outcome of atherosclerosis. A novel function for intestinal IDO in the fine-tuning of Trp metabolism with systemic effectson atherosclerosis was clarified by well-designed experiments. However, the interpreation of experimental data and in-depth analysis of gut microbiota need to be reconducted. The following main concerns should be considered before final acceptance.
1） As to "The absence of effects after a long period of HFD+HCD feeding in males was likely due to the significant decrease in Ido-1gene expression in the small intestines of control mice at 13 weeks compared to 8 weeks (Supp. Fig.2G).", why there is significant decrease of IDO-1 gene in control mice? What is the mechanism underlying?
2） As to "Interestingly, Ido-1 mRNA was markedly increased in control mice at 13 weeks compared to 8 weeks of HFD+HCD feeding (Supp. Fig. 3E), which most likely explain why the impact of intestinal IDO on plaque size in female mice was observed in advanced but not early atherosclerosis." What is the reason

for markedly increased in female control mice? It needs more explains for such observation, not likely.
3） A more deep and detailed analysis for gut microbiota data should be given. What is the main difference in the gut bacterial composition? How is the functional changes of gut microbiota in two groups.
4） As to “However, unexpectedly considering the availability of Trpfor the indole pathway in IEC IDOKO, lack of IDO in IECs led to a decrease in indole production, asassessed by low levels of fecal IAA (Supp. Fig. 10D), which most likely resulted from a decrease in bacteria catabolizing Trp to indole metabolites.” Authors need to identify the corresponding gut bacteria that influence the Trp catabolism under HFD condition for atherosclerosis disease and clarify their actions.
5） As to statistics, the normality of probability distribution should be tested with Kolmogorov-Smirnov test for each group of data, and the variance of probability distribution was tested with Brown-Forsythe test for determination of proper statistical analysis method.

Reviewer #4 (Remarks to the Author):

These studies show that deletion of intestinal indoleamine 2,3-dioxygenase 1 (IDO), the main enzyme catabolizing Trp in the gut enhances intestinal inflammation, impairs intestinal barrier function, and increases atherosclerosis along with T cell accumulation in plaques. This was accompanied by increased 5-hydroxytryptamine production and a decrease in indole metabolites. Inhibition of tryptophane hydroxylase 1 (TPH1) reduces intestinal inflammation 5-hydroxytryptamine production and reduces atherosclerosis and plaque inflammation. The authors suggest that they find a new role for intestinal IDO in Trp metabolism and atherosclerosis. A strength of the study is the use of a variety of approaches.
Major:
1) The authors state in the introduction that the specific role of intestinal IDO in atherosclerosis is unknown. This is true, because the role of intestinal IDO specifically in atherosclerosis has not been studied before. But, several papers have shown a role of systemic IDO expression in atherosclerosis, with results in line by those from those by the authors, or not. I would appreciate these papers to be cited in the introduction. The authors need to do a better job in pointing out the novelty of their manuscript. The discussion on this topic is also far too brief and would need to be more focused on cell specific roles of IDO. Could the diet used in the different studies with systemic IDO deficiency play an effect in the discrepancy of the atherosclerosis results as reported in earlier studies? Or would this be dependent on sex? Please discuss.
2) How do the authors exactly explain the difference between the effects of HCD and HFD in terms of IDO activity? It seems a bit unusual to combine two diets. Was the diet given to the mice a homogenous mix of HFD+HCD?
3) Supp Fig 2G. How do the authors explain the decrease in intestinal Ido-1 mRNA in male mice fed HCD+HFD for 13 weeks? It is logical that this decrease explains the lack of an effect of intestinal IDO on atherosclerosis. But why would intestinal Ido-1 mRNA increase in females fed HFD+HCD at 13 weeks compared to 8 weeks? These sex differences need to be studied in far more detail. Currently, the text on this topic is descriptive, without any mechanistic explanation.
4) Suppl Fig 5A-B and Suppl Fig 6. How do the authors explain the increase in IFNgamma and TNFalpha without an increase in Th1 cells? Would this simply be due to an increase in intestinal CD4+ and CD8+ T-

cells? What are the CD4+Tbet+ cells a percentage of? Same question for RORgammaT and Tregs. What happens to CD8+Tbet+ cells?
5) Fig 2G. The authors have observed that IDO deficiency in IECs increases CD3+ T cells in plaques and then carry out studies in mice with Rag1 deficiency to show that effects are dependent on lymphocytes. Have the authors also considered an effect of B cells? And how about effects on necrotic cores; were those also alleviated in the model with Rag1 deficiency? It seems to me that the plaques are much smaller in the Rag1 deficient mice, which may hamper interpretation on necrotic core size. Were mice from both sexes used?
6) How is the CD3+ area exactly calculated? Only in the intima? There seem to be several CD3+ cells also in the media.
7) How specific is LP533401 for 5-HT production? Please clarify.
8) What is the reason for only employing male mice for studies on LP533401? Initially the authors use mice from both sexes for their studies; in later studies they seem to stick to males.
9) I find the effects of antibiotics on lesion size a bit hard to interpret. What happens to the Ido mRNA expression in the intestine upon antibiotic use? The reason for this question that in males at 8 weeks, IEC IDO affects atherosclerosis, while this is not the case at 13 weeks when lesions are as big as in mice treated with antibiotics.
10) Another point is that the authors consistently measure plaque T cells in all studies but do not give a clear explanation as to how effects on IDO in the intestine would specifically increase T cells in plaques. What happens to blood T cells?
Minor:
11) Lay out of the figures is not uniform with many different fonts being used on the graphs. Please correct.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

This paper addresses the issue of the mechanisms whereby gut inflammation and dysbiosis might specifically contribute to atherosclerosis. The Authors show that the shift of intestinal tryptophan (Trp) metabolism from the production of microbiota-derived indole metabolites and 5-hydroxytryptamine (5-HT, serotonin) towards kynurenine production occurs in mice on high-fat diet (HFD) and is likely protective given that in HFD+HCD fed mice, the specific deletion of IDO in intestinal epithelial cells led to intestinal inflammation, impaired intestinal barrier, and exaggerated atherosclerosis along with augmented T lymphocyte accumulation within atheromatous plaques. This was associated with an increase in 5-HT production and a decrease in indole metabolites activating AhR. Inhibition of intestinal 5-HT production alleviated atherosclerosis and inflammation, while supplementation with an AhR agonist had the opposite effect. The Authors claim they have unraveled a novel function for intestinal IDO in the fine-tuning of Trp metabolism with systemic effects on atherosclerosis.

Response: It seems that this reviewer was mistaken in her/his statement that "*the inhibition of 5-HT production alleviated atherosclerosis while supplementation with an AhR agonist had the opposite effect*". Indeed, as clearly shown in Fig 4F, AhR agonist (Ficz) supplementation alleviated atherosclerosis, which indicates that Ficz exerted the same effect as did the inhibitor of 5-HT production, and not the opposite.

Comments

Low-density lipoprotein receptor (Ldlr-/-) mice, should be...-deficient mice

Response: we apologize for that, this has now been corrected

Several studies have also demonstrated a correlation between serotonin levels and the severity of atherosclerosis in animal models and human populations. Although the exact mechanisms through which serotonin contributes to atherosclerosis are still being investigated, elevated serotonin levels, either due to increased synthesis or impaired breakdown, have been associated with more extensive plaque formation and increased plaque vulnerability. The key question remains elucidating the environmental factors affecting if and how serotonin exerts pro-inflammatory, pro-thrombotic, and pro-proliferative effects that can contribute to the development and progression of atherosclerosis. In this work the Authors found that IDO1 may regulate serotonin production in that IDO1 deficiency in intestinal epithelial cells was associated with high levels serotonin production contributing to atherosclerosis. However, as N-acetylserotonin, a tryptophan metabolite produced along the serotonin pathway, acts as a positive allosteric modulator of IDO1 and thus increases kynurenine-mediated AhR activation (PNAS 117 (7) 3848-3857) – it needs to be assessed whether IDO1 activity, not modified in the gut upon blockade of TPH1, is instead induced by serotonin outside the intestinal epithelial cells, thus contributing to atherosclerosis. Indeed, in the context of atherosclerosis, the increased activity of IDO1 can contribute to the formation of unstable plaques and promote plaque inflammation. The authors have previously shown that IDO1 regulates immune homeostasis in atherosclerosis and colitis through IL-10 inhibition via the production of kynurenines, such that exogenous supply of kynurenines promoted atherosclerosis in IDO1-deficient mice and its blood levels predict death and recurrent myocardial infarction in

patients with coronary artery disease. Here, kynurenine administration failed to increase plaque size in Ldlr-/- IEC IDO KO suggesting that the main pathway responsible for atherosclerosis was not dependent on Kyn or Kyn-derived metabolites. The authors need to explain such a discrepancy and the contingency of kynurenine's molecular activity upon the different experimental settings. Was IDO1 induced in MOMA2+ macrophages within the atherosclerotic plaques in male Ldlr-/- IEC IDOKO mice?

Response: we fully agree with the reviewer that several previous studies showed the association between serotonin levels and CVD, although the role of serotonin in atherosclerosis has not been yet properly investigated. In our study, we showed that blocking intestinal 5 HT production markedly reduced intestinal, but also peripheral, serotonin levels (see Fig. 3A and supp. Fig 8D), which alleviated atherosclerosis likely due to a decrease in inflammatory cell (macrophages and lymphocytes) accumulation within the plaques (see Fig. 3C-D).

We believe that the reviewer's comment that the possible induction of IDO activity outside the gut after TpH1 inhibition could explain the observed effect on atherosclerosis is unlikely since, as in the gut (see supp. Fig. 8F), no significant differences of the ratio Kyn/Trp were observed in the plasma between controls and TpH1 inhibitor-treated mice. This new result has now been added as supp. Fig. 8G. Thus, the anti-atherogenic effect observed after the inhibition of intestinal 5-HT production was unlikely due to the induction of IDO activity within or outside the gut.

In the revised version of our manuscript, we have addressed the apparent discrepancy between our previous results on IDO (Cell Metab 2015 Sep 1;22(3):460-71) and the current results, in the Introduction and Discussion sections, as also recommended by the Reviewer#2. Please note that in our prior work (Cell Metab 2015 Sep 1;22(3):460-71), we showed an effect of **kynurenic acid and not kynurenine** in experimental models of atherosclerosis. Studies from other groups have shown that blood kynurenic acid levels predict death and recurrent myocardial infarction in patients with coronary artery disease. Also, please note that in the present study, we did not observe any effects of kynurenine despite its efficient supplementation in IEC IDO KO mice as shown in Supp. Fig. 8A. The main reason for this apparent discrepancy is that in our previous study, we showed a pro-atherogenic effect of IDO in mice with **global IDO deletion**. This pro-atherogenic effect was attributed to IDO expression in myeloid cells, involving the production of kynurenic acid. Moreover, the experimental condition used in that study could not allowed us to examine the specific role of intestinal IDO, since mice were **fed HCD and not HFD+HCD**. Importantly, as shown in Fig. 1A, we clearly showed that only HFD markedly increased intestinal IDO, **but not HCD**, which means that in the Cell Metab paper, HCD alone had little effect on intestinal IDO expression. In the present study, we used **IEC IDO KO mice** and **littermate controls** fed **HFD+HCD**, which allowed us to specifically address, for the first time, the role of intestinal IDO in atherosclerosis. This is an important question which brings a significant advance and knowledge, since Trp metabolism in the gut is specific insofar as serotonin and microbiota-produced indoles are mainly produced in the gut. Thus, IDO can exert different effects according to the cell and/or tissue where it is expressed. All these clarifications have now been added in the Introduction and Discussion sections pages 3 and 12.

We believe that the reviewer's comment about a possible induction of IDO in macrophages in IEC IDOKO mice could rather explain, at least in part, the observed decrease in fecal indole levels in IEC IDOKO mice, compared to controls (Supp. Fig. 12C). Indeed, we observed a decrease in indole production in IEC IDO KO mice compared to controls, whereas Trp was supposed to be available for indole and 5-HT pathways as a result of Kynurenine production blockade in the absence of IDO in

IECs. The likely explanation is a decrease in indole-produced bacteria and/or an induction of IDO in gut leukocytes including macrophages. To test this latter hypothesis, we performed a new series of experiments in which we FACS-sorted CD45+ cells from the small intestines of controls and IEC IDO KO mice fed HFD+HCD for 8 weeks, and assessed IDO expression in these cells. As shown in the new supp. Fig. 1E, IDO expression was significantly increased in CD45+ cells isolated from the small intestines of IEC IDO KO mice compared to littermate controls, which likely resulted from gut inflammation in IEC IDO KO mice (see Fig. 2A-C and supp. Fig.5). However, it is noteworthy that this induction was not important, since the IDO activity (Kyn/Trp) in the small intestines of IEC IDOKO mice compared to controls was still markedly dampened (see Fig. 1E), which indicates the importance of intestinal cells for IDO-mediated Trp metabolism in the gut.

The absence of IDO in IECs led to a significant increase in 5-HT production and plaque size in Ldlr-/- IEC IDOKO. Blockade of TPH1 was associated with a reduction in plaque size in the aortic sinus and in the thoracic aorta. However, to prove that intestinal 5-HT exerts pro-inflammatory and pro-atherogenic effects, as the authors claim, 5-HT needs to be administered under conditions of TPH1 deficiency. Moreover, a mechanistic explanation as to why 5-HT production increases in conditions of IDO-deficiency would be needed, considering the complex interplay between the IDO/Kyn and the 5-HT pathways at different body sites.

Response: please note that the main scope of our study was to investigate the role of the intestinal IDO on systemic atherosclerosis, since we used a murine model of specific IDO KO in the intestinal cells, and we also inhibited the serotonin production in the gut, the major source of body 5-HT. Regarding the explanation for why the serotonin increased in IEC IDOKO mice, the likely reason as stated page 8, is that Trp was more available for the 5-HT pathway as a result of Kyn pathway blockade in the absence of the intestinal IDO. Regarding the anti-atherogenic effect of blocking intestinal 5-HT production, we found this effect not only in wild type Ldlr-/- mice, but also in IEC IDOKO Ldlr-/- mice (see Fig. 3). Thus, the anti-atherogenic effect exerted by the inhibition of intestinal 5-HT production was independent of IDO pathway. Moreover, the 5-HT pro-atherogenic effect is in agreement with previously reported studies showing that serotonin has marked pro-inflammatory and pro-oxidation functions in inflammation-associated diseases (see for examples doi.org/10.1182/blood-2012-06-437392, https://doi.org/10.1053/j.gastro.2007.05.019).

This reviewer required us to supplement 5-HT in TPH1-deficient mice. Please notice that these mice were previously reported to exhibit serious fertility and developmental issues (doi: 10.1073/pnas.0606722104), which makes it challenging to crossbreed these mice with Ldlr-/- mice to study the effect of 5-HT supplementation in the context of atherosclerosis. However, to address this reviewer's request, we performed a new series of experiments in which Ldlr-/- mice were supplemented or not with 5-HT (see new supp. Fig.9). 5-HT supplementation in HCD-fed Ldlr-/-mice increased intestinal permeability, as assessed by augmented serum FITC-dextran after oral gavage, and also increased atherosclerosis without any significant changes in plasma cholesterol levels between the 2 groups (Supp. Fig.9C-D). Serum FITC-dextran levels were positively correlated with plaque size (Supp. Fig. 9E). Moreover, 5-HT blood levels were positively correlated with plaque size, indicating a pro-atherogenic role of 5-HT (Supp. Fig. 9F). Thus, both experiments using a TpH1 inhibitor in Ldlr-/- or Ldlr-/-IEC IDOKO mice or 5-HT supplementation indicate pro-inflammatory and pro-atherogenic effects of 5-HT.

The 16S rDNA sequencing of male Ldlr-/-IEC IDOKO and Ldlr-/-IEC IDO mice fed either HFD+HCD or NCD for 8 weeks revealed significant PCoA and relative abundances differences between the groups of mice associated with indoles production. The authors showed that dysbiosis caused by HDF feeding impacted gut inflammation and atherosclerosis. Who does what, and how? How the production of indoles are unexpectedly decreased under conditions of IDO1 deficiency in IEC? It has been demonstrated that the opposite holds true. The Authors shoud explain the discrepancy. Finally, is not particularly surprising AhR's role in atherosclerosis is not particularly surprising; the novelty would be clarifying how it is affected by either type of diet and/or specifically upon activation vs. inhibition of AhR activity.

Response: Please note that we can rule out an effect of the diet per se on the atherosclerotic phenotype since all mice used in our work were fed with the same diet, either HFD+HCD or HCD. For example, to study the impact of intestinal IDO in atherosclerosis, both IEC IDO KO mice and littermate controls were fed with the same diet HFD+HCD. The HFD was added to induce intestinal IDO activity (see Fig. 1A).

Concerning the comment about the decrease in indole production in IEC IDOKO mice (please see our response above).

We showed for the first time that the supplementation with an AhR agonist (Ficz) alleviated atherosclerosis by improving colon structure, as assessed by decreasing colonic histological scoring, as well as by reducing lymphocyte accumulation within the plaques (see Fig 4D-G). We furthermore specify the effects of AhR activation by microbiota-produced indoles on atherosclerosis by performing a new series of experiments in which we supplemented Ldlr-/- mice with indole derivatives (a mixture of IAA, IPA, and tryptamine). As shown in the new Fig. 5, indole derivatives supplementation decreased plaque inflammation and size without significant changes in plasma cholesterol between the groups. Indole supplementation was also associated with decreased leukocyte (CD45+) numbers in the intestine, particularly macrophages (CD64+), T (CD4+, CD8+) cells, and B (B220+) cells (Fig. 5B and Supp. Fig. 13C).

Reviewer #2 (Remarks to the Author):

Chajadine et al. investigate the role intestinal tryptophan catabolism in experimental atherosclerosis. They compared high fat (HFD), high cholesterol (HCD) and the combination with normal chow diet (NCD). One big issue arising in the study is that HFD and HCD were purified diets from Ssniff, Germany and the normal chow diet (A03, SAFE, France) was from a vendor in France. This issue makes all comparisons between HFD/HCD vs. NCD highly problematic, because the different diets show substantial differences (e.g. fiber, starch, choline, arginine, tryptophan, etc.) all known confounders for the microbiome, intestinal metabolites, vascular and immune phenotypes. This issue also leads to wrong interpretations on their microbiome and intestinal metabolite data. Nevertheless, several sub studies comparing genetic differences under one diet condition still are valid and interesting. They found that under HFD+HCD, the specific deletion of IDO in intestinal epithelial cells led to intestinal inflammation, impaired intestinal barrier, and exaggerated atherosclerosis along with augmented T lymphocyte accumulation within

atheromatous plaques. The phenotype was associated with an increase in 5-HT production and a decrease in indole metabolites. Of note, the Ldlr-/-Rag1-/-IEC IDOKO mouse studies demonstrate a clear role of lymphocytes in the IDO- atheromatous plaque phenotype.

Major critique:

- **All NCD vs. other group comparisons should be removed from the paper. Alternatively, the authors could repeat the whole study with an appropriated control diet.**

Response: we fully agree with this reviewer that HFD and HCD are purified diets and NCD is a non-purified diet. However, as shown in Fig. 1A regarding the increase in intestinal IDO activity under HFD compared to NCD, but this was also true when we compared the conditions HFD+HCD to HCD. Please note that both HFD and HCD were purified diets and from the same vendor Ssniff, which provides strong evidence that the increased intestinal IDO activity under HFD was unlikely dependent on the fact that the diet was purified or not, or on the source of the diet (France vs Germany). Furthermore, we showed in Fig. 1D that when we compared mice fed with the same HFD diet, but supplemented or not with soluble fibers (FOS), intestinal IDO activity was decrease after FOS supplementation, suggesting that fibers exert a negative regulation on intestinal IDO activity, indicating that HFD-induced increased intestinal IDO activity was caused by the low level of fibers in HFD. Also, please note that all major experiments to assess the role of gut tryptophan metabolism pathways in atherosclerosis were carried out in different groups of mice fed with the same diet, either HCD or HFD+HCD, excluding any potential bias due to the differences in diet composition according to the vendor.

- **The authors need to follow the ARRIVE guidelines. One important challenge in science is to improve the reproducibility. Therefore, precise reporting of methodological and technical details is essential, especially since it get more and more obvious that environmental factors are important modifiers of phenotypes. The authors do not provide sufficient information. E.g. the genetic strains including backcrossings is not reported; information on housing conditions (single vs. cage housing including the number of cages) is missing – cage effects have a pivotal role on microbiome; health status for the different lines need to be provided in the supplement, since different pathogen status is known to affect phenotypes, statistical details (e.g. outlier testing, testing for normal distribution, description which tests where used for which subset of experiments need to be described in each legend, etc.) are missing.**

Response: we have now added all the required information in the Material and Method section, as as requested by this reviewer. Regarding the cage effects, please note that the main experiments were repeated several times with mice in different cages, as for IEC IDO vs. IEC IDO KO (see Fig. 1H, Fig. 3E, Fig. 4C) and for TpH1 inhibitor (see Fig. 3B and Fig. 3E), which ruled out an eventual cage effect. The animal facility health certificate was provided as a supplementary table 1, and details of statistical analysis have been added in the statistical section and also detailed for each figure legend.

- **Along this line, the authors have performed experiments after antibiotics treatment. Except "We**

also subjected some mice to antibiotic (ATB) treatment as described before Ref. 45." and "male mice fed HFD+HCD using a broad-spectrum antibiotic cocktail (ATB) supplemented in the drinking water" no information on the type of antibiotics is given. It is not the task of the reviewer to hunt for this information.

Response: we apologize for that. we have now described antibiotic composition in the material and method chapter.

- **The microbiome data is superficial and not of high quality. Therefore, it should be deleted. Since the co-housing experiments are performed on the same diet (HFD+HCD), this set of experiment is fine.**

Response: we agree with this reviewer and we have now moved microbiome data to Supp. Fig. 11A and 11B as co-housing experiments shown in Fig. 4 is one the main experiments showing the involvement of gut microbiota in the phenotype.

- **Why do the authors sole report on C2 and C4 and not on C3?**

Response: we supposed that this reviewer meant by C1, C2, C3, and C4, the experimental conditions NCD, HCD, HFD, and HFD+HCD. With this regard, we have shown the results with HCD (either HCD or HFD+HCD), which is the only condition that allowed the development of atherosclerosis (in absence of cholesterol atherosclerosis does not develop in mice) and we added HFD in the diet in certain experiments to induce IDO activity within the gut.

- **What are the fecal IEC IDO KO SCFA levels?**

Response: we have now measured the levels of SCFA in feces of IEC IDOKO mice and littermate controls fed either NCD or HFD+HCD. The only difference that we observed was the decrease in fecal acetate levels in IEC IDO KO mice, compared to littermate controls fed NCD. Under HFD+HCD the levels of acetate markedly decreased with no significant differences between the 2 genotypes. This new result has now been added as Supp Fig. 11D.

- **Why do male Ldlr-/-Rag1-/-IEC IDO show already higher plaques size in a similar range as male IEC IDOKO in Fig. 1H?**

Response: please note that Ldlr-/-Rag1-/-IEC IDO mice have **lower, not higher,** plaque size compared to IEC IDOKO in Fig. 1H (mean plaque Ldlr-/-Rag$_1$-/-IEC IDO 69838 μm^2± 7919 (SEM) vs. Ldlr-/-IEC IDOKO 92297 μm^2± 5630 (SEM), p= 0.0595)

- **The authors found that AhR agonists (IAA, IAld, and tryptamine) were markedly decreased in Ldlr-/- IEC IDO KO and Ldlr-/- IEC IDO mice treated with ATB. Why did they perform the rescue experiment with FICZ and not a natural Ahr ligand such as e.g. IAA?**

Response: we have performed an additional series of experiments to examine the impact of indole supplementation on atherosclerosis, as requested by this reviewer. As shown in the new Fig. 5,

indole supplementation decreased plaque inflammation and size without significant changes in plasma cholesterol levels between the groups. Indole supplementation was also associated with decreased leukocyte (CD45+) numbers in the small intestines, particularly macrophages (CD64+), T (CD4+, CD8+) cells, and B (CD19+) cells (Fig. 5B and Supp. Fig. 13C). Thus, both experiments either using FICZ or indole derivatives as AhR agonists led to lowering atherosclerosis.

- **What is the role of FICZ on Th17/Treg ratio as well as IL-22?**

Response: we performed a new series of experiments to analyze CD4+ROR-γt+ cells known to produce IL17 and IL22 as well as Treg (Foxp3+) cells by flow cytometry in mice supplemented or not with AhR agonists. As shown in the new Supp. Fig. 14, no differences in lymphocyte polarization were observed between controls and indole-supplemented mice in spleens, MLNs, and small intestines. However, we did observe a higher differentiation towards more CD4+ROR-γt+ cells in Peyer's Patches (PPs) of indole-supplemented mice in comparison to controls (see new Supp. Fig. 14), suggesting a deviation towards the Th17 subset in PPs.

Reviewer #3 (Remarks to the Author):

The current manuscript reported the key role of intestinal tryptophan catabolism in the development and protection of atherosclerosis of disease. The shift of trytophan catabolism under HFD feeding significantly influenced the outcome of atherosclerosis. A novel function for intestinal IDO in the fine-tuning of Trp metabolism with systemic effectson atherosclerosis was clarified by well-designed experiments. However, the interpreation of experimental data and in-depth analysis of gut microbiota need to be reconducted. The following main concerns should be considered before final acceptance.

1） As to "The absence of effects after a long period of HFD+HCD feeding in males was likely due to the significant decrease in Ido-1gene expression in the small intestines of control mice at 13 weeks compared to 8 weeks (Supp. Fig.2G).", why there is significant decrease of IDO-1 gene in control mice? What is the mechanism underlying?

Response: The likely explanation for decreased IDO1 gene expression in the small intestines in males after 13 weeks of HFD+HCD, whereas in females the inverse tendency was observed (see supp. Fig 2G and 3F), is the difference in sex hormone regulation in males vs. females between 8 and 13 weeks of HFD+HCD feeding period. Indeed, we found a decrease in gene expression of estrogen receptors (*ESR1* and *ESR2 mRNA*), as well in that of their target gene (greb1), in females, and on the contrary a significant increase in these genes in males after 13 weeks of HFD+HCD (see new Supp. Fig. 3G). In particular, there was an inverse significant correlation between *IDO-1 mRNA* in small intestines and *ESR2 mRNA* expression (see new Supp. Fig. 3I), suggesting a negative regulation of ER on IDO expression in the gut, which is in agreement with a previous study in cancer cells (see https://doi.org/10.1158/2326-6066.CIR-16-0182). As there are no known estrogen response elements on the IDO1 promoter sequence, this implied an indirect effect of estrogens on IDO expression. We believe that identifying the exact mechanisms involved in this regulation is out of the scope of this study. All these clarifications have now been added to the discussion page 12.

2) As to "Interestingly, Ido-1 mRNA was markedly increased in control mice at 13 weeks compared to 8 weeks of HFD+HCD feeding (Supp. Fig. 3E), which most likely explain why the impact of intestinal IDO on plaque size in female mice was observed in advanced but not early atherosclerosis." What is the reason for markedly increased in female control mice? It needs more explains for such observation, not likely.

Response: please see our response above

3) A more deep and detailed analysis for gut microbiota data should be given. What is the main difference in the gut bacterial composition? How is the functional changes of gut microbiota in two groups.

Response: we have now focused our microbiota analysis on indole-producing bacteria that may explain microbiota-induced changes in atherosclerosis (see Fig. 4B and 4C). Among indole-producing bacteria (https://doi.org:10.1038/s41467-018-05470-4), *Parabacteroides distasonis* abundance decreased in IEC IDO KO mice and showed a positive correlation with fecal IAA levels and an inverse correlation with plaque size in control and Ldlr-/- IEC IDOKO mice fed HFD+HCD. These new results have been added as Supp. Fig. 12 E-G. Moreover, to assess the functional relevance of indoles on atherosclerosis, we performed a new series of experiments to examine the impact of indole supplementation including IAA on atherosclerosis. As shown in the new Fig. 5, indole supplementation decreased plaque inflammation and size without significant changes in plasma cholesterol levels between the groups. Indole supplementation was also associated with decreased leukocyte (CD45+) numbers in the intestine, particularly macrophages (CD64+), T (CD4+, CD8+) cells, and B (CD19+) cells (Fig. 5B and Supp. Fig. 13C).

4) As to "However, unexpectedly considering the availability of Trpfor the indole pathway in IEC IDOKO, lack of IDO in IECs led to a decrease in indole production, asassessed by low levels of fecal IAA (Supp. Fig. 10D), which most likely resulted from a decrease in bacteria catabolizing Trp to indole metabolites." Authors need to identify the corresponding gut bacteria that influence the Trp catabolism under HFD condition for atherosclerosis disease and clarify their actions.

Response: we have now analyzed the mechanisms that may explain the decrease in indole production in IEC IDO KO mice. Indeed, IEC IDOKO mice unexpectedly showed a decrease in indole production (see Supp. Fig. 12C), while this was supposed to be increased since Trp was available for this pathway, as a result of Kyn pathway blockade. The most likely explanation is a decrease in indole-produced bacteria and/or an induction of IDO expressed in gut leukocyte IDO, which can hijack Trp. In agreement with this hypothesis, we observed an increase in IDO expression in leukocytes (CD45+) isolated from the small intestines of IEC IDO KO mice compared to littermate controls after 8 weeks of HFD+HCD (see the new Supp. Fig. 1E). We also observed a decrease in the abundance of *Parabacteroides distasonis* involved in indole production (see new supp. Fig. 12E-G), which may explain, at least in part, the observed decrease in indoles in IEC IDO KO mice.

5) As to statistics, the normality of probability distribution should be tested with Kolmogorov-

Smirnov test for each group of data, and the variance of probability distribution was tested with Brown-Forsythe test for determination of proper statistical analysis method.

Response: we have performed Kolmogorov-Smirnov test to assess the normality of the data and Brown-Forsythe test to test the variance. In cases where the data did not pass the normality test, non-parametric statistical tests have been applied. The exact statistical tests used have now been added to each figure legends.

Reviewer #4 (Remarks to the Author):

These studies show that deletion of intestinal indoleamine 2,3-dioxygenase 1 (IDO), the main enzyme catabolizing Trp in the gut enhances intestinal inflammation, impairs intestinal barrier function, and increases atherosclerosis along with T cell accumulation in plaques. This was accompanied by increased 5-hydroxytryptamine production and a decrease in indole metabolites. Inhibition of tryptophane hydroxylase 1 (TPH1) reduces intestinal inflammation 5-hydroxytryptamine production and reduces atherosclerosis and plaque inflammation. The authors suggest that they find a new role for intestinal IDO in Trp metabolism and atherosclerosis. A strength of the study is the use of a variety of approaches.
Major:
1) The authors state in the introduction that the specific role of intestinal IDO in atherosclerosis is unknown. This is true, because the role of intestinal IDO specifically in atherosclerosis has not been studied before. But, several papers have shown a role of systemic IDO expression in atherosclerosis, with results in line by those from those by the authors, or not. I would appreciate these papers to be cited in the introduction. The authors need to do a better job in pointing out the novelty of their manuscript. The discussion on this topic is also far too brief and would need to be more focused on cell specific roles of IDO. Could the diet used in the different studies with systemic IDO deficiency play an effect in the discrepancy of the atherosclerosis results as reported in earlier studies? Or would this be dependent on sex? Please discuss.

Response: In this new version of the manuscript, we cited the papers exploring the role of IDO in atherosclerosis using global IDO KO mice in the introduction chapter (page 3), as requested by this reviewer. We also better explain the apparent discrepancy between our previous results on IDO (Cell Metab 2015 Sep 1;22(3):460-71) and the current results, in the Introduction and Discussion sections, as also recommended by Reviewer#2. In our previous study (Cell Metab 2015 Sep 1;22(3):460-71), we showed an effect of kynurenic acid, which belongs to Kynurenine pathway, in atherosclerosis. Please note that in the present study, we did not observe any effects of kynurenine despite its efficient supplementation in IEC IDO KO mice as shown in Supp. Fig. 8A. The main reason for this apparent discrepancy is that in the previous paper, we showed a pro-atherogenic effect of IDO by in mice with global IDO deletion. We showed that this pro-atherogenic effect can be attributed to IDO expression in myeloid cells, involving the production of kynurenic acid. Moreover, the experimental condition used in that study could not allow us to examine the role of intestinal IDO since the mice were fed **HCD and not HFD+HCD**. Indeed, as shown in Fig. 1A, only high-fat diet, not HC, can induce intestinal IDO. In the present study, we used IEC IDO KO mice and littermate controls fed HFD+HCD, which allowed us to specifically address, for the first time, the role of intestinal IDO in atherosclerosis. This is an important question that brings significant advance and knowledge since Trp metabolism in the gut is specific insofar as serotonin and microbiota-produced indoles are mainly

produced in the gut. Thus, IDO can exert different effects according to the cell and/or tissue where it is expressed. All these clarifications have now been added in the Introduction and Discussion sections pages 3 and 11.

2) How do the authors exactly explain the difference between the effects of HCD and HFD in terms of IDO activity? It seems a bit unusual to combine two diets. Was the diet given to the mice a homogenous mix of HFD+HCD?

Response: HFD, but not HCD, contains a low content of fibers, which leads to a low SCFA production as shown in Supp. Fig. 1A. Moreover, the lack of SCFA, as butyrate, which has a negative impact on intestinal IDO gene expression (see doi: 10.3389/fimmu.2018.02838), led to the observed increase in IDO activity in the gut. Thus, by combining HFD (to induce intestinal IDO) with HCD (to induce atherosclerosis), we revealed the role of IDO in the gut. HFD+HCD diet was given as a homogenous diet, we have already published the effects of this diet on gut microbiota (see DOI:https://doi.org/10.1016/j.celrep.2023.113350)

3) Supp Fig 2G. How do the authors explain the decrease in intestinal Ido-1 mRNA in male mice fed HCD+HFD for 13 weeks? It is logical that this decrease explains the lack of an effect of intestinal IDO on atherosclerosis. But why would intestinal Ido-1 mRNA increase in females fed HFD+HCD at 13 weeks compared to 8 weeks? These sex differences need to be studied in far more detail. Currently, the text on this topic is descriptive, without any mechanistic explanation.

Response: The likely explanation for decreased IDO1 gene expression in the small intestines in males after 13 weeks of HFD+HCD, whereas in females the inverse tendency was observed (see supp. Fig 2G and 3F), is the difference in sex hormone regulation in males vs. females between 8 and 13 weeks of HFD+HCD feeding period. Indeed, we found a decrease in gene expression of estrogen receptors (*ESR1* and *ESR2 mRNA*), as well in that of their target gene (greb1), in females, and on the contrary a significant increase in these genes in males after 13 weeks of HFD+HCD (see new Supp. Fig. 3G). In particular, there was an inverse significant correlation between *IDO-1 mRNA* in small intestines and *ESR2 mRNA* expression (see new Supp. Fig. 3I), suggesting a negative regulation of ER on IDO expression in the gut, which is in agreement with a previous study in cancer cells (see https://doi.org/10.1158/2326-6066.CIR-16-0182). As there are no known estrogen response elements on the IDO1 promoter sequence, this implied an indirect effect of estrogens on IDO expression. We believe that identifying the exact mechanisms involved in this regulation is out of the scope of this study. All these clarifications have now been added to the discussion page 12.

4) Suppl Fig 5A-B and Suppl Fig 6. How do the authors explain the increase in IFNgamma and TNFalpha without an increase in Th1 cells? Would this simply be due to an increase in intestinal CD4+ and CD8+ T-cells? What are the CD4+Tbet+ cells a percentage of? Same question for RORgammaT and Tregs. What happens to CD8+Tbet+ cells?

Response: the increase in inflammation was likely explained by an increase in lymphocyte numbers (shown now in Supp. Fig. 6A), as no differences in terms of Th polarization (as expressed in percent of CD4+) including ROR-γt+, Tbet+, and Foxp3+ T cells were observed (see Supp. Fig. 6B). The same was also true for CD8+ cells (data not shown).

5) Fig 2G. The authors have observed that IDO deficiency in IECs increases CD3+ T cells in plaques and then carry out studies in mice with Rag1 deficiency to show that effects are dependent on lymphocytes. Have the authors also considered an effect of B cells? And how about effects on necrotic cores; were those also alleviated in the model with Rag1 deficiency? It seems to me that the plaques are much smaller in the Rag1 deficient mice, which may hamper interpretation on necrotic core size. Were mice from both sexes used?

Response: we performed B cell staining using B220+ antibody within the plaques and found very few positive cells within the plaques. In lymphocyte-deficient mice (RAG1KO), no differences in the necrotic core size were observed between the 2 groups (i.e. Control vs IEC IDO KO) (mean necrotic core surface 1186 $\mu m^2 \pm$ 192 (SEM) vs. 1705 $\mu m^2 \pm$ 258 (SEM), p= 0.11), which is in agreement with the absence of difference in plaque size in RAG1KO mice (see Fig. 2I). Please note that we used both sexes to study the effects of intestinal IDO in atherosclerosis. However, to study the mechanisms involved, we used only males because the phenotype was observed at an earlier time point (8 weeks), compared with females in which changes in phenotype occurred at a later time point (13 weeks). We hope that this reviewer will agree that, given the huge amount of experiments already done, we cannot replicate all the results in both males and females at 8 weeks and 13 weeks of HFD+HCD.

6) How is the CD3+ area exactly calculated? Only in the intima? There seem to be several CD3+ cells also in the media.

Response: CD3+ area was delimited only in the intima, where inflammation develops in the arterial wall, as previously shown (see. for example, J Clin Invest. 2001 Jul 15; 108(2): 251–259).

7) How specific is LP533401 for 5-HT production? Please clarify.

Response: LP533401 is a specific inhibitor of TpH1 (ref#46 in the text), an isoform mainly expressed in the gut, responsible for more than 90 % of whole body 5-HT (see for example doi.org:10.1016/j.pharmthera.2019.107423). Moreover, as shown in Fig. 3A, TpH1 inhibition by LP533401 was efficient as it led to a decrease of about 80 % of intestinal 5-HT production.

8) What is the reason for only employing male mice for studies on LP533401? Initially the authors use mice from both sexes for their studies; in later studies they seem to stick to males.

Response: we carried out the majority of the experiments in males. We hope that this reviewer can understand that considering the huge amount of the experiments already done, we cannot replicate all the experiments in both males and females.

9) I find the effects of antibiotics on lesion size a bit hard to interpret. What happens to the Ido mRNA expression in the intestine upon antibiotic use? The reason for this question that in males at

8 weeks, IEC IDO affects atherosclerosis, while this is not the case at 13 weeks when lesions are as big as in mice treated with antibiotics.

Response: please note that the absence of effects in ATB-treated mice was not related to a decrease in IDO1 expression in treated IEC IDO mice, compared to non-treated mice, since we observed rather a tendency to increased IDO expression (data not shown). The main reason for the disappearance of the effect is that ATB treatment depleted microbiota, which is involved in the phenotype, whereas non-treated IEC IDO after 13 weeks of HFD+HCD showed a decrease in IDO1 expression, which likely explained the absence of effects in this group. Moreover, the involvement of microbiota in the phenotype was also supported by co-housing experiments (Fig. 4C), showing that microbiota exchange abolished the differences in plaque size.

10) Another point is that the authors consistently measure plaque T cells in all studies but do not give a clear explanation as to how effects on IDO in the intestine would specifically increase T cells in plaques. What happens to blood T cells?

Response: we have now added as a new Supp. Fig. 7B the analysis of blood lymphocyte numbers observed in male IEC IDO and IEC IDO KO after 8 weeks of HFD+HCD. We observed an increase in circulating numbers of CD4+, CD8+, and CD19+ cells in IEC IDO KO mice, compared to littermate controls, which likely contributed to an increased lymphocyte accumulation within the plaque (see Fig. 2G).

Minor:

11) Lay out of the figures is not uniform with many different fonts being used on the graphs. Please correct.

Response: we apologize. This has now been corrected

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The present manuscript certainly contains many data elements, but it does not provide an overarching framework in which all the pieces fit together to allow the reader to derive a clear and cohesive picture. The pieces of evidence are connected by a myriad of merely speculative mechanistic bridges, or at most, historical ones, such that controlled conditions are lacking, or oversimplifications are present, such as the generic use of antibiotics. The microbiota study is still uninformative and remains superficial, particularly when dealing with indole-producing bacteria.

Reviewer #2 (Remarks to the Author):

The authors have addressed most of my concerns. Especially the new experiments with the mix of indole metabolites increase the quality of the paper. I still have 2 issues, which need to addressed:

1. The authors state that the insoles are given '(2 mg/ml diluted in drinking water, Sigma-Aldrich)'. First are all indoles given at 2 mg/ml? And second, they should express it as mg/kg/d.

2. The issue of chow vs. purified diets: The reviewer agrees that some (important) comparisons are made with a consistent protocol. However, because statistical tests are performed between NCD and purified groups, e.g. in Figure 1, the reader cannot assess whether the difference is due to the chow/purified diet or to the change in fat/cholesterol. The authors need to make this clear (either by removing NCD or by stating it clearly in the text!)

Reviewer #3 (Remarks to the Author):

Authours have made satisfactory reponses and revisions on the comments and questions raised by reviewers. I would like to recommend acceptance for this submission.

Reviewer #4 (Remarks to the Author):

The authors have done a good job in responding to some, though not all of my points.
In relation to my previous point 2, it would be good if the authors explain the reason for using HFD+HCD in the text and not only in the response to the reviewers.
Also please indicate at first mention in the text that CD4+ and CD8+ cells are T cells and CD19+ cells are B cells.
Related to my previous point on Th1 cells, it would be helpful if the authors provide representative figures of flow cytometry plots that are quantified in Suppl Fig 14. Could the authors also indicate the absolute numbers of T cell subsets shown in Suppl Fig 14 (as such correcting for the differences shown in

Suppl Fig 13)? This would be more convincing; otherwise differences on T cell subsets can only be inferred. The same accounts for data in Suppl Fig 6.

Response to reviewers

Reviewer #1 (Remarks to the Author):

The present manuscript certainly contains many data elements, but it does not provide an overarching framework in which all the pieces fit together to allow the reader to derive a clear and cohesive picture. The pieces of evidence are connected by a myriad of merely speculative mechanistic bridges, or at most, historical ones, such that controlled conditions are lacking, or oversimplifications are present, such as the generic use of antibiotics. The microbiota study is still uninformative and remains superficial, particularly when dealing with indole-producing bacteria.

Response: we have performed several complementary approaches to investigate the impact of each tryptophan catabolic pathway on atherosclerosis. As such, we used a mouse model with a specific deletion of IDO in intestinal epithelial cells to investigate the role of IEC IDO in atherosclerosis. In this regard, we showed that intestinal IDO deletion has a detrimental role, in part due to increased serotonin production (see Fig. 3 and Supp. Fig.10A). Indeed; inhibition of serotonin production in this model but also in Ldlr-/- mice alleviated inflammation and atherosclerosis (see Fig. 3). In summary this paper untangled the intricate and complex crosstalk between Trp-dependent catabolic pathways to decipher the role of each intestinal Trp-catabolic pathway in atherosclerosis.

We would like to emphasize that the impact of microbiota on the phenotype was not studied only through the use of antibiotics but also with the use of other experimental studies such as cohousing (see Fig.4C and Supp. Fig. 12B).

Regarding indole-producing bacteria, we would like to highlight that we have identified *Parabacteroides distasonis* as bacteria which the relative abundance decreased in IEC IDO KO mice, and showed a positive correlation with fecal IAA levels and an inverse correlation with plaque size (see Supp. Fig. 12E-G). Moreover, we studied the functional impact of bacteria-produced indoles showing their anti-atherogenic effects (see Fig. 5 and Supp. Fig. 13).

Reviewer #2 (Remarks to the Author):

The authors have addressed most of my concerns. Especially the new experiments with the mix of indole metabolites increase the quality of the paper. I still have 2 issues, which need to addressed:

1. The authors state that the insoles are given '(2 mg/ml diluted in drinking water, Sigma-Aldrich)'. First are all indoles given at 2 mg/ml? And second, they should express it as mg/kg/d.

Response: yes, all indole derivatives are given at 2 mg/ml. The indole metabolites were added to the drinking water, this is why we expressed it as a concentration. As shown in Fig. 5A, we checked the effective supplementation of indole derivatives.

2. The issue of chow vs. purified diets: The reviewer agrees that some (important) comparisons are made with a consistent protocol. However, because statistical tests are performed between NCD and purified groups, e.g. in Figure 1, the reader cannot assess whether the difference is due to the chow/purified diet or to the change in fat/cholesterol. The authors need to make this clear (either by removing NCD or by stating it clearly in the text!)

Response: This has now been clearly stated in the manuscript page 4.

Reviewer #3 (Remarks to the Author):

Authours have made satisfactory reponses and revisions on the comments and questions raised by reviewers. I would like to recommend acceptance for this submission.

Response: we are happy that we have satisfactorily addressed all this reviewer's comments.

Reviewer #4 (Remarks to the Author):

The authors have done a good job in responding to some, though not all of my points.
In relation to my previous point 2, it would be good if the authors explain the reason for using HFD+HCD in the text and not only in the response to the reviewers.

Response: this is indicated page 5 of the manuscript

Also please indicate at first mention in the text that CD4+ and CD8+ cells are T cells and CD19+ cells are B cells.

Response: this is now mentioned in the text pages 5-6

Related to my previous point on Th1 cells, it would be helpful if the authors provide representative figures of flow cytometry plots that are quantified in Suppl Fig 14. Could the authors also indicate the absolute numbers of T cell subsets shown in Suppl Fig 14 (as such correcting for the differences shown in Suppl Fig 13)? This would be more convincing; otherwise differences on T cell subsets can only be inferred. The same accounts for data in Suppl Fig 6.

Response: Please notice that we presented Th subsets in Supp. Fig. 14 and Supp. Fig. 6: Th1 (T-bet+), Treg (Foxp3+), and Th17 (RoR-γt+) as % of CD4+ cells, which is classically used to assess a shift toward a particular Th subset. Supp. Fig. 14 indicates that there is no shift toward particular Th subsets except for Th17 (RoR-γt+) in Peyer Patches (PP). As recommended by this reviewer, we have added representative flow cytometry plots to Suppl. Fig. 14 and 15.